THE ROLE OF THE MACROPHAGE IN THE HOST RESPONSE TO BACTERIAL ENDOTOXINS

RICHARD J. ULEVITCH, JOHN C. MATHISON and PETER S. TOBIAS

Department of Immunology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA

INTRODUCTION

Gram-negative bacillary infections in man are occurring with increasing frequency in hospitalized patients, and are associated with an unacceptably high mortality rate. In patients with multiple injuries, the likelihood of complications due to Gram-negative infection is high, and despite advances in antibiotic therapy, the prognosis for these patients is bleak. These patients often develop hypotensive shock, disseminated intravascular coagulation, and metabolic abnormalities, all of which are resistant to standard treatment and together contribute to the multiple organ failure observed in these patients. Unique to Gram-negative bacilli is the presence of a complex glycolipid in the outer cell membrane. This component, termed endotoxin or lipopolysaccharide (LPS), is now generally recognized as the bacterial product that is responsible for initiating the biochemical changes leading to shock, DIC, and death.

Despite the early recognition of an important role for LPS in Gram-negative septicemia, it has been difficult to determine the exact mechanisms of injury, in part because of the inability to identify the primary, injurious events occurring after exposure to LPS. In fact, based on *in vitro* studies, we are faced with too many possible mediators since LPS has been shown to activate multiple humoral and cellular mediation systems.[1] Nevertheless, attention has recently been focused on the macrophage as a source of mediators of the early biochemical changes induced by LPS that result in hypotension, DIC, and metabolic changes. The purpose of this report is to describe a number of mediators produced by LPS-treated macrophages that may participate in the host response to LPS, namely, a supernatant factor of LPS-treated macrophage (M), which suppresses adrenocortical steroidogenesis, and a membrane-bound procoagulant activity, which activates coagulation Factor X directly.

SUPPRESSION OF ADRENOCORTICAL STEROIDOGENESIS BY A MACROPHAGE PRODUCT

The therapeutic benefit of steroids in Gram-negative septicemia is controversial.[2,3] Related to this question is the possibility of abnormal adrenocortical function in septic patients.[4] Melby and Spink,[5] in 1958, suggested that adrenocortical function is normal in patients with bacteremic shock, but since then, a number of other reports have documented conflicting results. Hubay et al.[6] noted patients with adrenocortical insufficiency, Migeon et al.[7] described adrenocortical insufficiency in children with disseminated meningococcemia, and Sibbald et al.[8] reported that 5/26 patients with severe bacteremia demonstrated impaired adrenocortical responsiveness to ACTH. Other evidence for adrenocortical insufficiency derives from the findings of Berry and Smythe,[9] where evidence of decreased adrenal responsiveness to ACTH in mice injected with endotoxin was observed. Finally, the recent study of Keri et al.[10] suggested that soluble mediators might suppress adrenocortical function, since plasma from rabbits in shock as a result of live E. coli infusion suppressed steroidogenesis by ACTH-stimulated, explanted adrenocortical cells. This later study suggested that potential mechanisms of adrenocortical suppression in endotoxic shock should be examined further.

Recently, we examined the possibility that products of LPS-treated M could mediate suppression of adrenal steroidogenesis. Resident and peptone-elicited murine peritoneal macrophages (PEM) were placed in plastic flasks for 2 hr at 37°C in RPMI 1640 with 5 percent fetal calf serum (FCS). After washing to remove the non-adhered cells, one half of the flasks were treated with sufficient macrophage-activating factor (MAF, also known as macrophage cytotoxicity factor) for 4 hr to render the macrophages cytotoxic for tumor cells.[11] The MAF-containing solution was removed, followed by several rinses and replenishment of fresh medium. Then one half of the MAF-treated and untreated cells were exposed to 10 ug/gm Salmonella minnesota Re595 LPS for 18 hr. As controls, flasks without cells were incubated with medium, MAF, and LPS. The macrophage supernatants and control media were then centrifuged, filtered, (0.22 um) and stored at -20°C. Rabbit adrenocortical cells were isolated by collagenase digestion and maintained (as adherent cells) for 3 days in 6-mm-diameter plastic culture dishes in HEPES-buffered MEM with 15 percent FCS. The following additions were made to these adrenocortical cells: (a) 78 ul

MEM-HEPES-FCS; (b) 30 ul macrophage supernatant, control medium, or MEM-HEPES; and (c) 12 ul ACTH dissolved in MEM-HEPES. The final concentration of ACTH in the wells was 10 mU/ml. After 18 hr at 37°C, the adrenocortical supernatants were harvested, and steroids were determined by a fluorometric assay. As shown in Table 1, steroidogenesis was not suppressed by control medium + LPS or by supernatants from macrophages that were not exposed to LPS. However, supernatants from LPS-treated resident and peptone-elicited macrophages did suppress steroid production by approximately 40 percent, and as much as 80-90 percent suppression was observed with supernatants from LPS-treated macrophages that had received prior exposure to MAF. In control experiments we have obtained evidence that (a) the macrophage-supernatant-induced suppression is not a result of LPS carried over from the macrophage culture and (b) the macrophage supernatants do not degrade or inactivate ACTH and do not interfere with the assay of fluorogenic steroids. These results provide support for an LPS-induced macrophage factor(s) that suppresses adrenocortical suppression in

TABLE 1. SUPPRESSION OF ACTH-INDUCED STEROIDOGENESIS BY SUPERNATANTS OF LPS-TREATED PERITONEAL EXUDATE MACROPHAGES[a]

Additions			Fluorogenic Steroid Production (% of maximum response)
Supernatants:			
Macrophage	LK	LPS	
Resident	-	+	59 ± 9
Peptone	-	+	59 ± 11
Resident	+	+	23 ± 5
Peptone	+	+	14 ± 7
Control Sup[b]	±	-	92 ± 9
Control Medium	±	±	98 ± 9
MEM + HEPES + FCS[c]			100

[a] See text for experimental details
[b] Flasks without cells were treated with medium ± LK followed medium ± LPS in the same manner as the macrophage cultures.
[c] Medium used for culture of adrenocortical cells.

Gram-negative sepsis. A number of preliminary experiments have also been performed to characterize the biochemical properties of the M-derived factor. These data are summarized in Table 2. The details of these findings have been recently published.[12]

TABLE 2. PROPERTIES OF MACROPHAGE FACTOR THAT SUPPRESSES ADRENOCORTICAL STEROIDOGENESIS

Treatment	Result
Dialysis using 12,000-14,000 mw cutoff tubing	Activity retained
Ultrafiltration (10,000 mw cutoff)	Activity retained (suppressive activity not observed in ultrafiltrate)
Repeated freeze thawing and storage at -20°C	Stable
Exposure to pH 4 or pH 11 30 min at 37°C	Stable
70°C, 30 min	Stable
100°C, 5 min	Labile
HPLC with Bio-SIL TSK 250	Activity in 40-60 kD range

Studies of the mechanism of ACTH-induced steroidogenesis have provided many details of the molecular basis ACTH action (reviewed in ref. 13). It is known that ACTH binds to plasma membrane receptors, which, as suggested by the recent studies of Buckley and Ramachandran,[14] number approximately 4,000 per cell and have a Kd of 1.4 nM. Calcium and cAMP serve as important second messengers in the response to ACTH. Although a 2-log greater ACTH concentration is required to induce increased cytoplasmic levels of cAMP than is required for induction of steroidogenesis, the dose-response curves for ACTH-induced protein kinase activity and steroidogenesis have been shown to be nearly superimposable.

Since conversion of cholesterol to pregnenolone (the rate-limiting step in steroidogenesis) is blocked by cycloheximide but not actinomycin D, it has been suggested that synthesis of a labile protein is required for cholesterol side chain cleavage to occur. Conversion of pregnenolone to the various steroid products involves, among other things, increased requirement for reducing equivalents (NADPH), a complex array of enzymes that are partitioned in mitochondria and in the smooth endoplasmic reticulum, and mechanisms for shuttling steroid intermediates between the mitochondria and cytosol. We have observed that supernatants from LPS-treated macrophages that suppress the steroidogenic response to ACTH also produce an equivalent degree of suppression of the response to cholera toxin as well as dibutyryl cyclic AMP. Cholera toxin is known to bind to GM1 gangliosides of the plasma membrane with resulting stimulation of adenylate cyclase, whereas dibutyryl cyclic AMP enters the cell and apparently directly stimulates protein kinsase activity. These results suggest that macrophage factor-induced adrenocortical suppression does not result from inactivation of the ACTH receptor or from a block of adenylate cyclase activity, but rather from disruption of steps distal to formation of cAMP.

It has been well appreciated that corticosteroids have marked effects on cells involved in the induction of the immune response and in mediating the inflammatory response. Most recently Snyder and Unanue[15] showed that therapeutic dosages of corticosteroids suppressed interleukin-1 production by LPS-stimulated murine peritoneal exudate cells. Of further interest is the possible connection between cells of the immune and nervous system, suggested by reports demonstrating regulation of the <u>in vitro</u> antibody response by neuroendocrine hormones. Finally, in view of the potential role of endorphins in endotoxic shock, it is clear that previously unappreciated interrelationships between cells of the immune/inflammatory systems and neuroendocrine systems may be of importance in regulating the host response to bacterial endotoxins. The data reported in this paper demonstrating suppression of adrenocortical steroidogenesis by a macrophage-derived product add a new regulatory pathway that may well influence the host response to LPS. Increased understanding of this phenomenon should provide insight into the mechanisms of endotoxic shock.

MACROPHAGE-ASSOCIATED PROCOAGULANT ACTIVITY

After an intravenous LPS injection the liver is the tissue that contains the majority of the tissue-bound LPS.[16] When radiolabled LPS is injected and electron microscopy and autoradiography of tissue sections is performed, LPS localization is observed in the cytoplasm of hepatic macrophages (H-M).[16] Other studies using electron microscopy to identify LPS in tissue sections have described similar findings,[17,18] and thus this M population represents a major cellular target of LPS.

One of the consistent histologic findings following a single injection of LPS is fibrin deposition in the liver microcirculation[19] and association of the fibrin with the H-M (John Mathison, personal communication). These data prompted us to determine if the hepatic M can respond to LPS by producing mediators that might play an important role in LPS-induced fibrin deposition. To accomplish this, we first needed a source of purified H-M, so we used a combination of mechanical and enzymatic disaggregation of rabbit liver to prepare isolated H-M.[20] These cells can be maintained in culture for up to 10 days without outgrowth of other cell types, and they respond to LPS in a number of different ways, including the selective induction of enzymes.[20] Addition of LPS prepared from Salmonella minnesota Re595 to the cultured macrophages also induces a procoagulant activity (PCA) detectable in cell lysates when assayed in a one-stage clotting assay.[21] The H-M PCA was detected in cells treated with as little as 10 ng/ml of Re595 LPS, and evidence of increased activity was apparent as early as 3 hr after LPS addition, with a maximal response occurring between 7 and 12 hr after LPS.

Previous reports have described the association of tissue thromboplastin with LPS-treated macrophages.[22] The PCA found in LPS-treated H-M has a number of biochemical properties that clearly distinguish it from thromboplastin. These properties are summarized in Table 3. Of interest, however, is the observation that combination of the H-M PCA with tissue thromboplastin results in a 10-fold greater activity than expected from the separate activity of the H-M PCA and the tissue thromboplastin. These data are shown in Table 4 and suggest, together with the biochemical characteristics of the H-M PCA, that the H-M PCA is an enzyme with properties similar to those of activated coagulation

TABLE 3. PROPERTIES OF THE HEPATIC MACROPHAGE PROCOAGULANT ACTIVITY (H-Mo PCA)

H-M PCA	Tissue Thromboplastin
Heat labile 56°C, 30 min	Heat stabile, 56°C, 30 min
DFP sensitive	DFP resistant
DASA sensitive[a]	?
Corrects Factor VII deficient plasma	Does not correct Factor VII deficient plasma
Activates Factor X to Xa	Activates Factor VII

[a] H-M were pretreated for 15 min at 37° with 5 uM diazonium salt of sulfanilic acid (DASA).

TABLE 4. EFFECT ON COMBINING H-Mo LYSATES AND BRAIN THROMBOPLASTIN ON THE TOTAL PCA

		Heat Treated (56°C)	
	Untreated	20 min	40 min
H-M Lysate[a]	10,000 (100)[b]	3,200 (32)	800 (8)
Tissue Factor[c]	9,800 (98)	9,600 (95)	8,800 (88)
Combination (1:1)	96,000 (960)	34,000 (340)	9,500 (95)

[a] H-M lysates (approximately 5 x 10^5 cells/m^{10} from cultures pretreated for 24 hr with 1 ug LPS/ml medium)

[b] Numbers are representative of a typical experiment and represent PCA in milliunits with neat thromboplastin standard equal to 100,000 milliunits. The numbers in parentheses are percent of activity present relative to the activity of the lysate PCA in NRP being 100 percent.

[c] Rabbit brain thromboplastin standard (1:10)

Factor VII. In this regard Curtiss et al.[23] have recently reported the production of a PCA by LPS-treated human peripheral blood mononuclear cells, which was neutralized by antibody to human Factor VII. This PCA appeared to be membrane bound, could be dissociated by EDTA treatment, and activates purified

Factor X to Factor Xa. Thus, this LPS-induced human moncyte PCA appears to be similar to that first identified in the rabbit H-M. This suggests that the LPS-treated rabbit H-M may express Factor VII or VIIIa on the plasma membrane.

Chapman et al.[24] have also recently described the induction of a PCA associated with murine peritoneal exudate macrophages (PEM), which acts by activating Factor X. Thus, different sources of M can produce a PCA in response to LPS, which is most likely Factor VII or VIIa. This membrane-bound PCA, together with tissue thromboplastin, may produce the initiating signal for localized fibrin deposition and DIC observed after LPS injection.

ACKNOWLEDGMENTS

This is publication Number 3120-IMM from the Department of Immunology, Scripps Clinic and Research Foundation. This work has been supported by U.S.P.H.S. grants AI-15136, and GM-28485. R.J.U. is a recipient of a R.C.D.A. AI-00391 and J.C.M. is a recipeint of U.S.P.H.S. Grant No. AI-19965.

REFERENCES

1. Morrison, D.C. and Ulevitch, R.J. (1978) The effects of bacterial endotoxins on host mediation systems. Am. J. Path. 93:527-617.

2. Schumer, W. (1976) Steroids in the treatment of clinical septic shock. Ann. Surg. 184:333-341.

3. Kreger, B.E., Craven, D.E., and McCabe, W.R. (1980) Gram-negative bacteremia. IV. Re-evaluation of clinical features and treatment in 612 patients. Am. J. Med. 68:344-355.

4. Beisel, W.R. and Rapoport, M.I. (1969) Inter-relations between adrenocortical functions and infectious illness. N. Engl. J. Med. 280:541-546.

5. Melby, J.C. and Spink, W.W. (1958) Comparative studies on adrenal cortical function and cortisol metabolism in healthy adults and in patients with shock due to infection. J. Clin. Invest. 37:1791-1798.

6. Hubay, C.A., Weckesser, E.C., and Levy, R.P. (1975) Occult adrenal insufficiency in surgical patients. Ann. Surg. 181:325-332.

7. Migeon, C.J., Kenny, F.M., Hung, W., and Voorhess, M.L. (1967) Study of adrenal function in children with meningitis. Pediatrics 40:163-183.

8. Sibbald, W.J., Short, A., Cohen, M.P. and Wilson, R.F. (1977) Variations in adrenocortical responsiveness during severe bacterial infections. Ann. Surg. 186:29-33.

9. Berry, L.J. and Smythe, D.S. (1961) Effects of bacterial endotoxin on metabolism. IV. Renal function and adrenocortical activity as factors in the nitrogen excretion assay for endotoxin. J. Exp. Med. 114:761-778.

10. Keri, G., Parameswaran, V., Trunkey, D.D., and Ramachandran, J. (1981) Effects of septic shock plasma on adrenocortical cell function. Life Sciences 28:1917-1923.

11. Schrieber, R.D., Altman, A., And Katz, D.H. (1982) Identification of a T-cell hybridoma that produces large quantities of macrophage-activating factor. J. Exp. Med. 156:677-689.

12. Mathison, J.C., Schreiber, R.D., LaForest, A.C., and Ulevitch, R.J. (1983) Suppression of ACTH-induced steroidogenesis by supernatants from LPS-treated peritoneal exudate macrophages. J. Immunol. 130:2757-2762.

13. Rasmussen, H. (1981) Hierarchical Control in Calcium and cAMP as Synarchic Messengers. John Wiley and Sons, New York, p. 178.

14. Buckley, D.I. and Ramachandran, J. (1981) Characterization of corticotropin receptors on adrenocortical cells. Proc. Natl. Acad. Sci. USA 78:7431-7435.

15. Snyder, D.S. and Unanue, E.R. (1982) Corticosteroids inhibit murine macrophage in expression and interleukin 1 production. J. Immunol. 129:1803-1805.

16. Mathison, J.C. and Ulevitch, R.J. (1979) The clearance, distribution and cellular localization of lipopolysaccharide (LPS) in rabbits. J. Immunol. 123:2133-2143.

17. Nolan, J.P. (1981) Endotoxin, reticuloendothelial function and liver injury. Hepatology 1:458-465.

18. Levy, E., Slusser, R.J., and Ruebner, B.H. (1968) Hepatic changes produced by a single dose of endotoxin in the mouse. Am. J. Path. 52:477-490.

19. Coalson, J.J., Benjamin, B.A., Archer, L.T., Beller, B.K., Spaet, R.H., and Hinshaw, L.B. (1978) A pathologic study of Escherichia coli shock in the baboon and the response to adrenocorticosteroid treatment. Surg. Gynecol. Obstet. 147:726-736.

20. Maier, R.V. and Ulevitch, R.J. (1981) The response of isolated rabbit hepatic macrophages to lipopolysaccharide. Circ. Shock 8:1-18.

21. Maier, R.V. and Ulevitch, R.J. (1981) The induction of a unique procoagulant activity in rabbit hepatic macrophages by bacterial lipopolysaccharides. J. Immunol. 127:1596-1600.

22. Rivers, R.P.A., Hathaway, W.E., and Weston, W.C. (1977) The endotoxin-induced coagulant activity of human monocytes. Brit. J. Haem. 30:311-316.

23. Curtiss, L.K., Tsao, B.P., Fair, D.S., and Edgington, T.S. (1983) T cell instructed expression of monocyte Factor VII activity by lipopolysaccharide (LPS). Fed. Proc. 42:957 (Abst. No. 3895).

24. Chapman, H.A., Jr., Vavrin, Z., and Hibbs, J.B., Jr. (1983) Coordinate expression of macrophage procoagulant and fibrinolytic activity in vitro and in vivo. J. Immunol. 130:261-266.

DISCUSSION PERIOD WITH DR. ULEVITCH

UNKNOWN: What therapeutic implications do you see in regards to the patient in septic shock?

DR. ULEVITCH: That is a very difficult question. I think that right now our goal is to take the information from these in vitro studies and try to develop ways to intervene in vivo. For example, one can assume that there is a macrophage factor that suppresses steroidogenesis, which may play a role in sensitizing a patient or an experimental animal to endotoxin. If that factor could be obtained in pure form, you could devise a number of ways to intervene. But at the present time, I think there are not any clear methods.

What I hope to do is to stimulate people who are working with patients or other animal models to try to use this information in their studies and see if it is relevant.

DR. WUSTROW: I have two questions. First, have you tried to use LPS-resistant animals, like the C3H mice, which do not respond to LPS? Second, a factor has been described called tumor necrotic factor. Is that factor similar to what you have shown?

DR. ULEVITCH: There is at least one (and possibly two) strain of mice that is essentially resistant to the toxic effects of lipopolysaccharide. One of the strains is a C3H/HeJ. We have used macrophages from those mice, and they do not produce this steroidogenesis suppressive factor in response to LPS. They do, however, produce it in response to Listeria. So that is consistent.

We have also shown that this acute-phase protein that somehow modulates, or interacts with LPS in serum, cannot be induced in the HeJ mouse by LPS, but can be induced by other non-specific inflammatory stimuli. Everything we see fits with what is known about that mouse strain and its unresponsiveness.

In terms of tumor necrotic factor, we don't know if it is related or not. There is a whole series of activities now being identified as elaborated from macrophages. In addition to what I have described, there is a factor that has actually been purified now from LPS-stimulated macrophages, which can produce the insulin resistance seen in septic patients or in experimental models of endotoxic shock. It also acts on the fat cell by inhibiting the synthesis of enzymes that convert glucose to lipids. What the relationships are between all of these factors is as yet undetermined; however, they may be critical in mediating injury.

DR. NETA: In your studies on steroidogenesis suppressive factor from macrophages, did you also have a chance to look at the properties of your lymphokine preparation in the similar system?

DR. ULEVITCH: Lymphokines alone are not effective. The lymphokine preparation we have is a crude supernatant, although it is from a T-cell hybridoma. It is very rich in the lymphokine known as macrophage-activating factor, or MAF, but we are not prepared to say which lymphokine is involved. I think everybody who works in this field is faced with the problem of having lots of activity and nothing they can get their hands on in terms of pure molecules.

DR. NETA: I agree, but did you test Bob Schriber's purified interferon/MAF preparations?

DR. ULEVITCH: It is likely that the MAF is, in fact, gamma interferon, and we have not tested any pure preparations of gamma interferon.